

Claims:

1. Method for the determination of the identity of at least one nucleotide in a RNA-molecule comprising the steps of:
  - 5 (a) providing a single stranded form of the RNA-molecule;
  - (b) hybridising an oligonucleotide primer binding to a predetermined position of the RNA molecule;
  - (c) performing at least one primer extension reaction, whereby the oligonucleotide primer is extended on the RNA-molecule through incorporation of at least  
10 one nucleotide by the action of a RNA dependent polymerase;
  - (d) detecting the presence or absence of incorporation, thereby indicating the nucleotide identity of the RNA molecule in the relevant position;  
whereby step (c) to (d) optionally are repeated.
2. Method according to claim 1, whereby step (c) to (d) are repeated.
- 15 3. Method according to claim 1 or 2, whereby the incorporated nucleotide(s) is (are) recorded.
4. Method according to claim 1-3, whereby the presence or absence of incorporation is indicated by the presence of a detectable moiety.
5. Method according to claim 4, wherein the detectable moiety is removed or neutralized in step (d) after the detection.  
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6. Method according to claim 1-5, whereby the primer extension reaction results in the release of a residue molecule.
7. Method according to claim 6, whereby the primer extension reaction results in the release of a PPi molecule only upon incorporation of a nucleotide.
- 25 8. Method according to claim 7, wherein step (c) is performed by including enzymes, comprising luciferase, apyrase, and ATP-sulfurylase, and reagents to detect the release of PPi to trigger the release of light.
9. Method according to claim 1-8, whereby at least one nucleotide is labelled, such as fluorescently or radioactively, thereby allowing the detection of step (c) to be

performed by means of detecting the presence or absence of a labelled nucleotide.

10. Method according to claim 9, whereby the label on the labelled nucleotide is cleavable.

5 11. Method according to any one of the preceding claims, whereby the detection of step (c) is performed by means of detection of a change in physical properties of the RNA-molecule.

12. Method according to any one of the preceding claims, whereby the RNA dependent polymerase is an RNA dependent DNA polymerase or an RNA dependent RNA polymerase.

13. Method according to claim 12, whereby the RNA dependent RNA polymerase originates from any RNA virus or bacteriophage, such as bacteriophage phi 6.

14. Method according to claim 12, whereby the RNA dependent DNA polymerase is a RT-polymerase.

15 15. Method according to claim 14, whereby the RT polymerase is chosen from the group comprising: HIV-1 RT, M-MuLV RT, AMV RT, RAV2 RT, Thermoscript AMV RT, Superscript II M-MuLV RT, Tth DNA polymerase.

16. Method according to any one of the preceding claims, whereby a mixture of RNA dependent polymerases is added to the reaction mixture of step (a).

20 17. Method according to any one of the preceding claims, whereby the extension reaction is performed at a temperature ranging from 28 to 70 °C.

18. Method according to any one of the preceding claims, whereby the pH of the extension reaction solution is in the interval from 7.6 to 8.6, preferably from 8.0 to 8.4.

25 19. Method according to any one of the preceding claims, whereby the concentration of deoxynucleotides is in the interval from 1 µM to 1 mM.

20. Method according to any one of the preceding claims, whereby the salt concentration of the reaction mixture is in the interval from 10 to 100 mM.

0 21. Method according to any one of the preceding claims, wherein the oligonucleotide primer is a DNA primer.

22. Method according to claim 21, whereby the nucleotide is the deoxynucleotide dATP, which further is exchanged for the analogue alpha-S-dATP.
23. Method according to claim 1-20, wherein the oligonucleotide primer is a RNA primer.
- 5 24. Method according to claim 23, whereby the nucleotide ATP is exchanged for the analogue alpha-S-ATP
25. Method according to any one of the preceding claims, whereby a RNA-secondary structure reducing reagent, preferably chosen from the group comprising T4 Gene 32 Protein, retroviral nucleocapsid protein, actinomycin D,  
10 glycerol, methyl mercury hydroxide, methoxyamine-bisulfite, DMSO, spermidine, formamide, SSB (single stranded binding protein) and blocking primer, is included in the extension reaction.
26. Method according to any one of the preceding claims, whereby the RNA molecule is subjected to an RNA amplification prior to the extension reaction.
- 15 27. Method according to claim 26, whereby the nucleotide rITP is exchanged for rGTP in the amplification.
28. Method according to any one of the preceding claims, whereby the RT polymerase essentially lacks RNase H activity.
29. Method according to any one of the preceding claims, wherein the oligonucleotide primer is immobilised to a solid phase or wherein the RNA molecule is  
20 captured to a solid phase by an immobilised oligonucleotide.
30. Method according to any one of the preceding claims, whereby the quantity of the RNA-molecule is determined by measuring the intensity of the incorporation signal and comparing it to a reference.
- 25 31. Kit for performing the nucleotide identification of claim 1-30, comprising in separate vials a RNA dependent polymerase, nucleotides, necessary enzymes for a sequencing-by-synthesis reaction, and optionally other necessary reagents.
32. Kit according to claim 31, which further comprises a RNA quantity reference sample.

33. Method for determining the sequence of a ribonucleic acid molecule comprising the steps of;

- a) providing a single-stranded form of said ribonucleic acid molecule;
- b) hybridizing a primer to said single stranded form of said ribonucleic acid molecule to form a template/primer complex;
- c) enzymatically extending the primer by the addition of an RNA dependent polymerase and a mixture of nucleotides and a derivative of said nucleotides, wherein the derivative of said nucleotide comprises a label linked to a nucleotide via an optionally cleavable link and wherein the proportion in the mixture between the nucleotides and the derivative of said nucleotide is within the range of 1-60%, 1-50%, 1-40%, 1-30%, or 1-20%, preferably in the range of 5-60%, 5-50%, 5-40%, 5-30%, or 5-20%, or more preferably in the range of 10-60%, 10-50%, 10-40%, 10-30%, or 10-20%.

d) determining the type of nucleotide added to the primer;

34. Method according to claim 33, wherein the label is neutralized after step d) by the addition of a label-interacting agent or by bleaching, preferably by photo-bleaching.

35. Kit comprising, in separate compartments, a mixture of natural nucleotides and a derivative of said nucleotides according to step c) of claim 33, and at least one of the following components; an RNA dependent polymerase, a reducing agent, a carrier, a capping agent, an apyrase, an alkaline phosphatase, a PP-ase, a single strand binding protein or the protein of Gene 32, for performing the method according to claim 33-34.